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AMYLASE AND GLYCOGENOLYSIS IN AMPHIBIAN DEVELOPMENT [1]

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Since the carbohydrate reserves in amphibian eggs are mainly stored as glycogen (Gregg, 1948), attempts have been made to estimate its utilization during development as a function of glycogen breakdown. The most reliable data show that glycogen level begins to decrease when time of gastrulation starts (Brachet and Needham, 1935; Gregg, 1948). This has been confirmed by microchemical (Heatley and Lindahl, 1937; Jaeger, 1945) as well as histochemical methods (Woerdemann, 1933; Raven, 1935) which have also shown that glycogenolysis is stronger in those cells involved in the morphogenetic movements of gastrulation. Besides, the determination of the respiratory quotient (R.Q.) has given values concordant with those results. It is true that Barth (1946) has found a constant value of about 0.9; but Brachet (1934) and Boell (1955) have reported low values during segmentation, with a tendency to increase up to a value close to 1 at the time of gastrulation.

Less is known about the egg enzymes involved in glycogenolysis. Some evidence was reported indicating that glycogen breakdown could be accomplished through phosphoroclastic (Cohen, 1954; Gregg *et al.*, 1964) as well as through amylolytic pathways (see Urbani, 1962, for a review of the subject). The most outstanding news reported by the Italian author was the description of a β-amylasic activity in eggs of *Rana esculenta* and *Bufo vulgaris*.

Nothing is known, however, about glycogenolytic enzymes in *Bufo arenarum*. The limited information we possess on glycogen utilization during development agrees with the above reported results. Thus, a glycogen loss in eggs could be detected after the onset of gastrulation (Barbieri and Gil, 1962); and the R.Q. values were found to increase from about 0.6 during segmentation, up to 1 at the time of gastrulation (Legname and Barbieri, 1962).

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The present paper has two purposes. The first is to report some additional data about glycogen contained in the eggs of the toad. The second is to present our first observations about the properties and biological role of an amylase found in the same material.

Material and Methods

Biological material

Oocytes of *Bufo arenarum* obtained by injection of pituitary glands preserved according to Pisanó (1956) were artificially fertilized. Development was allowed to proceed in 10% amphibian Ringer's solution without bicarbonate at laboratory temperature. Prior to homogenization, the jelly coat was dissolved by ultraviolet irradiation or by a neutralized thioglycolic acid solution.

Cellular fractionation

The eggs were homogenized, unless otherwise stated, with two volumes of chilled 0.25 *M* sucrose solution containing 0.001 *M* ethylenediamine-tetraacetic acid (disodium salt). The homogenization was carried out in an ice-cooled Potter type glass homogenizer. In order to establish the localization of glycogen and amylase, cellular fractions were isolated by differential centrifugation in Sorval RC-2 or Christ Universal refrigerated centrifuges. The homogenate was centrifuged, 20 minutes each time, at 1500 *g*, yielding a pellet containing nuclei, yolk platelets and pigment granules (fraction I), and at 10,000 and 25,000 *g*, sedimenting the mitochondria (fractions II and III). The remaining supernatant will be referred to as fraction IV.

Chemical methods

As a mild procedure for the extraction of glycogen, the $HgCl_2$ method of Mordoh *et al.* (1966) was adopted. A comparison of $HgCl_2$-extracted glycogen with cold trichloracetic acid-extracted glycogen, prepared from the same batch of eggs, has shown that they do not differ from the standpoint of the properties considered in this paper. As a standard method for the estimation of glycogen the phenol-sulfuric acid method as described by Dubois *et al.* (1956) was employed. The iodine reaction was performed in the presence of calcium chloride, according to Krisman (1962). Liver glycogen from adult specimens of the same species was used as a standard for both methods, and, in some experiments, it was extracted from the same females wherefrom the eggs used for analysis had been taken.

For the determination of amylase activity the reaction mixture contained, except where otherwise stated, 1.2 mg. of glycogen, 0.2 *M* phosphate buffer at pH 7.2, 0.1 *M* NaCl and 0.02 ml. of crude enzyme preparation, in a total volume of 0.08 ml. Incubation time was 20 minutes at 37° C., and the reaction was stopped by heating 2 minutes at 100° C. Reducing power was determined according to Somogyi and Nelson (Ashwell, 1957).

Absorption spectra of the color reactions with iodine were determined with a Beckman DU spectrophotometer, and the photometric readings with the phenol-sulfuric and Somogyi-Nelson methods were performed with a Spectronic 20 (Bausch & Lomb).

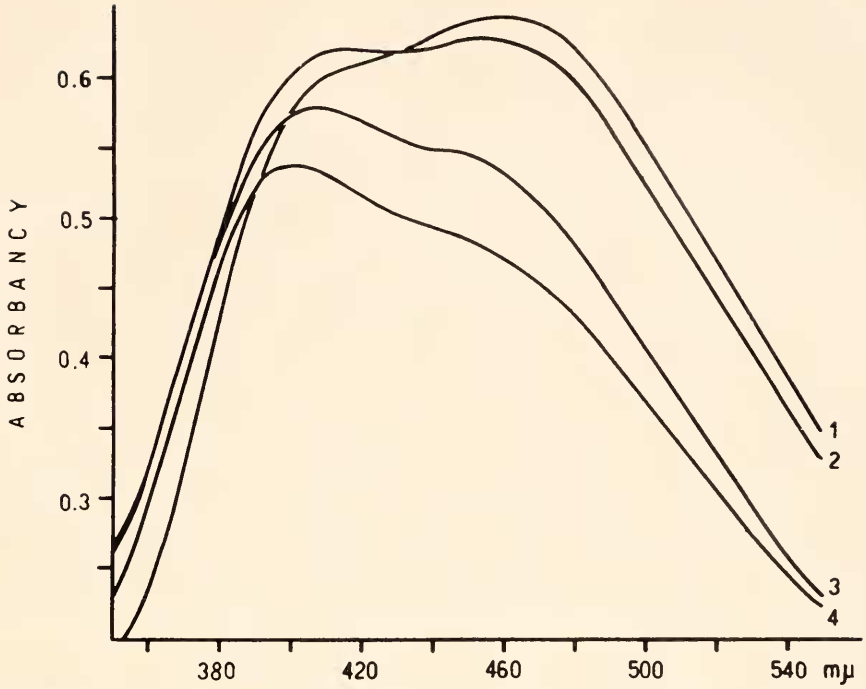

FIGURE 1. Absorption spectra of four glycogen preparations (0.05%) in the presence of iodine reagent. 1: unfertilized eggs; 2: rat liver; 3: toad liver; 4: tail bud embryos.

RESULTS

Glycogen

Egg glycogen dissolved in water never exhibits the milky appearance of the same concentration of liver particulate glycogen. Differential centrifugation of egg glycogen in aqueous solution, as well as its precipitation with varying concentrations of ethanol, allowed a rough estimate of its extensive polydispersity.

When studying the glycogen-iodine complex, egg glycogen was compared with liver glycogen of the same species as well as with rat liver glycogen prepared by

TABLE I

Changes in glycogen as shown by the iodine color reaction

Clutch	Stage	Iodine phenol-sulfuric	E_{400}/E_{460}
1	Second cleavage	0.98	1.09
	Neural fold	0.73	1.21
2	Unfertilized egg	1.02	0.98
	Tail bud	0.70	1.06
3	Unfertilized egg	1.09	0.97
	Tail bud	0.85	1.17

the same procedure. We found that the absorption spectrum of rat liver glycogen in the presence of iodine reagent presented, in addition to the absorption maximum at about 460 mμ as reported by Krisman (1962), a second λ_{max} at 410 mμ. Both maxima have about the same height and are separated by a slight depression (Fig. 1).

With respect to the toad, a significant difference could be detected between liver and oocyte glycogen. While liver glycogen showed a λ_{max} at about 400–410 mμ, oocyte glycogen presented a λ_{max} between 450 and 460 mμ (Fig. 1).

The affinity of glycogen for iodine was expressed by the ratio of the amounts of glycogen as determined by the iodine and the phenol-sulfuric acid methods, the value of this ratio being taken as 1 for the standard (Krisman, 1962). In the case of the iodine method, estimations were based on the average of extinctions at 400 and 460 mμ. The values of this ratio varied among different batches, but were generally slightly higher for oocytes than for liver glycogen.

When glycogen is extracted from developing eggs after gastrulation the absorption maximum shifts from 460 towards 400 mμ (Fig. 1). This displacement is expressed in Table I by the ratio of absorbancies at 400 and 460 mμ (E_{400}/E_{460}). Besides, the values of the ratio iodine/phenol-sulfuric indicate a fall of glycogen affinity for iodine over 20%. This change of affinity could also be detected in four glycogen fractions arbitrarily isolated by fractionated precipitation with ethanol (Fig. 2).

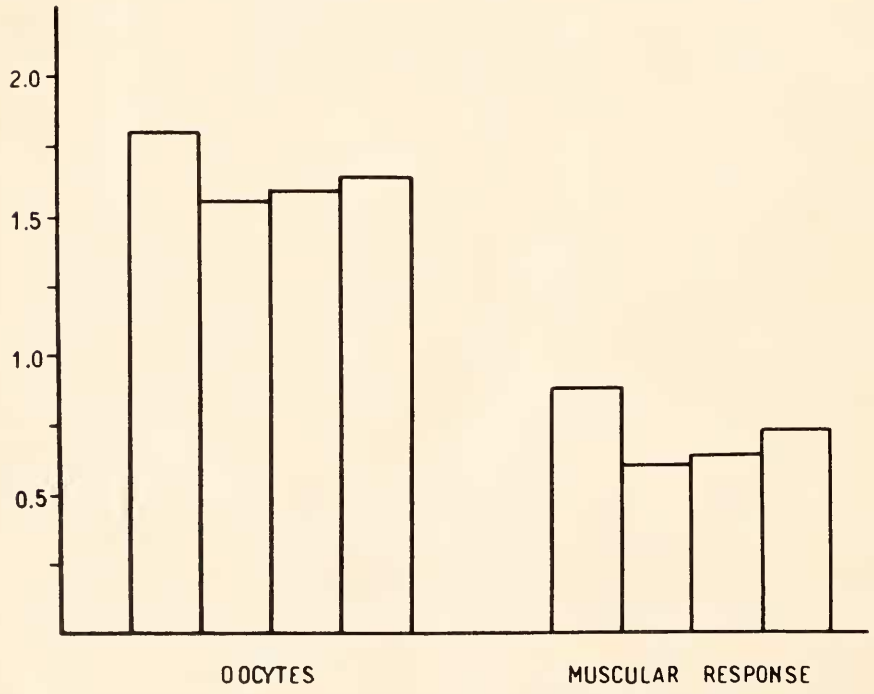

FIGURE 2. Histograms expressing the values of the iodine/phenol-sulfuric ratio in four glycogen fractions precipitated with 20, 30, 40 and 67% ethanol (reading from left to right).

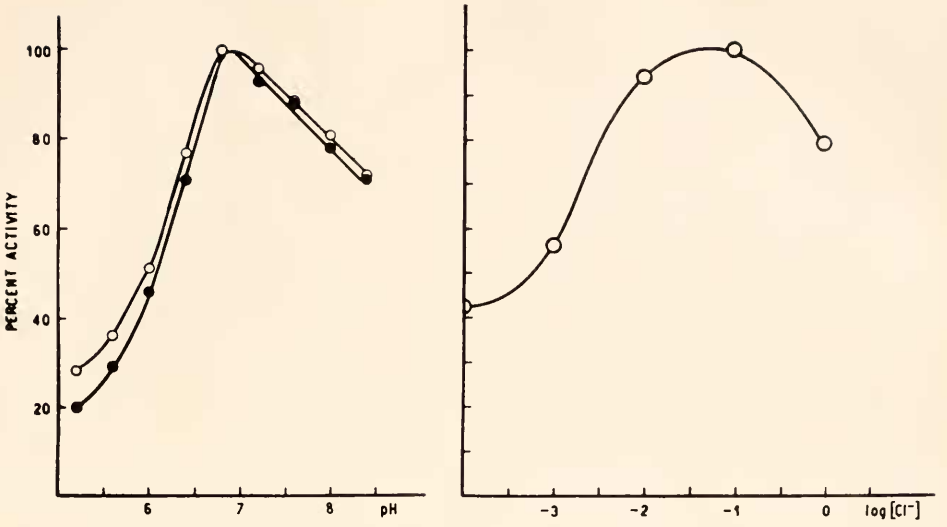

FIGURE 3 (left). pH optimum. Open circles: egg amylase; solid circles: mammalian salivary amylase.

FIGURE 4 (right). Effect of chloride on enzyme activity.

In connection with a point to be discussed later, it is important to add that egg glycogen does not seem to be associated to particles sedimenting as fraction I. In our working conditions, less than 10% of the total amount of glycogen could be detected in that fraction.

Amylase activity

The homogenates of *B. arenarum* eggs degrade glycogen in the same conditions as α-amylase. Thus, the pH of maximum activity is about 6.8 and it decreases sharply out of the range 6.4–7.8 (Fig. 3). The enzymatic activity is enhanced by chloride ions with an optimum concentration between 0.01 and 0.1 *M* (Fig. 4). Zn^{2+} is inhibitory and 10^{-5} *M* $HgCl_2$ showed no effect on the enzymatic activity (Table II). A Km value of 3.7 mg./ml. was found.

TABLE II

Effects of $ZnCl_2$ and $HgCl_2$ on amylase activity

Additions	Final concentration (*M*)	Enzyme activity*	
		Fraction I	Salivary amylase
$ZnCl_2$	10^{-3}	87	79
	10^{-2}	70	74
	10^{-1}	0	8
$HgCl_2$	10^{-5}	98	—

* Activity without salts taken as 100.

TABLE III

Recovery of amylase activity as a function of the procedure followed in the preparation of fraction I

Preparation	Enzyme activity (%)	
	Sediment	Supernatant
In 0.25 *M* sucrose	79	21
In 0.25 *M* sucrose washing once	2	98
In distilled water	29	71

After fractionation of oocyte homogenates, most of the activity appears localized in fraction I (nuclei, yolk and pigment granules), though in a very labile fashion. In fact, it suffices to wash the pellet only once with sucrose solution, or to homogenize the eggs with water, in order to loose the enzymatic activity of fraction I (Table III). We cannot decide as yet to which particles the enzyme is associated, but it does not seem to be linked to yolk platelets of major or medium size,

TABLE IV

Recovery of amylase activity from 0.25 M sucrose breis as a function of centrifugal force

Centrifugation	Enzyme activity (%)	
	Sediment	Supernatant
1500 g/20 min.	84	16
500 g/30 sec.	2	98

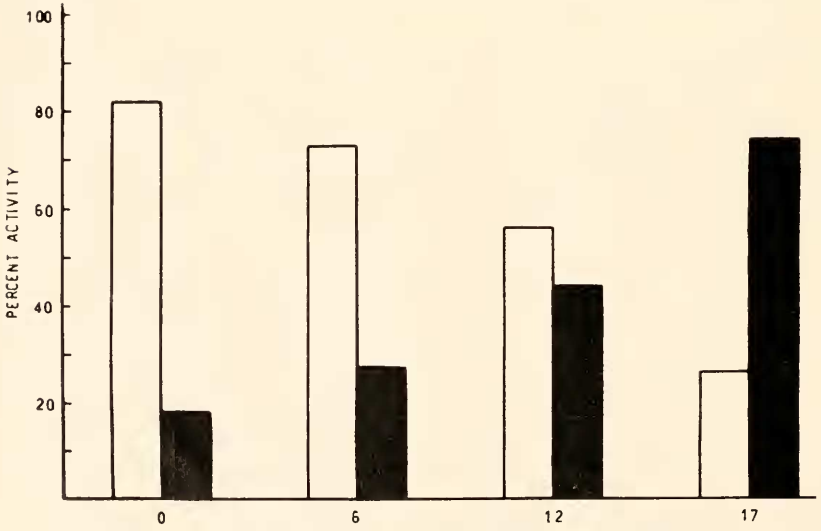

FIGURE 5. Enzyme activity of fractions I (nuclei-yolk-pigment) and IV (supernantant) as a function of developmental age. Abscissa, embryonic stages: 0, unfertilized eggs; 6, early cleavage; 12, gastrulation; 17, tail bud.

TABLE V

Activity of fraction I on glycogen as shown in vitro

Minutes of incubation	Reducing power (%)	Iodine/phenol-sulfuric	E_{400}/E_{460}
0	3	1.15	0.91
5	25	1.08	0.97
12	59	0.97	1.05
20	100	0.83	1.13

The incubation mixture contained: 2.4 mg. of glycogen and 0.05 ml. of enzyme in 0.004 *M* maleic acid-KOH buffer and 0.1 *M* NaCl (total volume 0.6 ml.).

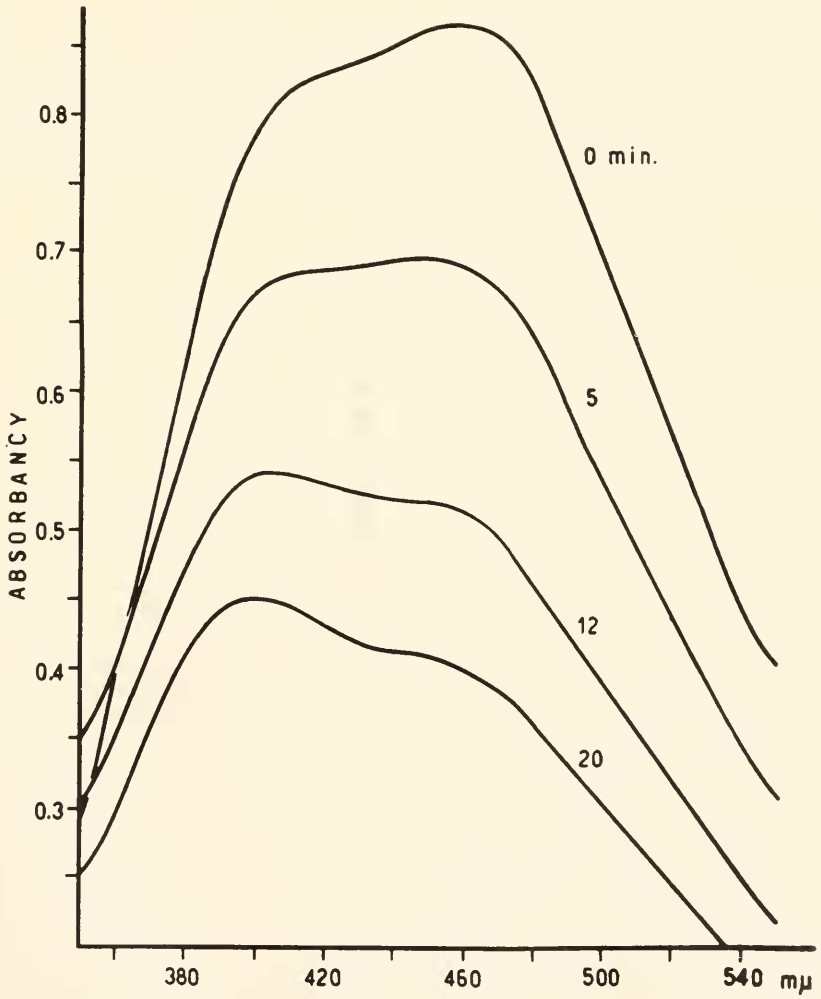

FIGURE 6. Glycogen breakdown by fraction I of unfertilized eggs, as shown by the iodine method. Incubation times are indicated in minutes.

since after sedimentation of most of the yolk mass at a low centrifugal field (500 *g* for 30 seconds) the activity remains in the supernatant (Table IV). Neither does it seem to be associated with the pigment granules, since an important part of these particles also sediments at 500 *g*.

Although the level of amylase activity remains constant up to the end of the neurula stages, the intracellular localization of the enzyme seems to change as a function of embryonic development (Fig. 5). While the activity of fraction I, which is the most important in the oocytes, decreases during development, the activity of fraction IV, *i.e.,* of the supernatant, increases at the same time. It is after gastrulation when the major part of the enzymatic activity appears in fraction IV at the expense of fraction I. The properties of this "soluble amylase" were found to be the same as those described for the enzyme linked to fraction I of the unfertilized egg.

The *in vitro* attack of glycogen by the enzyme of fraction I was also studied with the iodine color reaction. After incubating oocyte glycogen with a suspension of fraction I of the same origin, it was isolated by the $HgCl_2$ method and analyzed with the iodine reagent. The data collected in Table V and Figure 6 show that the optical properties of the glycogen iodine complex change in the same way after glycogen breakdown *in vivo* or *in vitro*.

Discussion

The iodine color reaction has proved useful to get some additional information about glycogen utilization in amphibian eggs. Let us point out first that the different absorption spectra reported for the toad and rat liver glycogens agree with previous observations indicating that the properties of the glycogen-iodine complex depend on the origin of the polysaccharide (Schlamowitz, 1951; Manners, 1957). Much more knowledge will be needed before the real basis of these differences may be understood; some evidence, however, is available showing that the iodine reaction reflects some structural aspects of the glycogen molecule. Thus, it has been shown that the value of the specific absorptivity coefficient is a function of the chain length and the value of the λ_{max} is related to the degree of branching of the polysaccharide (Swanson, 1948; Thoma and French, 1960; Archibald *et al.,* 1961; Bailey and Whelan, 1961). This holds also true for the reaction performed following the technique used in this paper (Krisman, 1962).

We have pointed out that after gastrulation, when a consumption of glycogen can be already detected, a fall of its affinity for iodine as well as a shift of the λ_{max} from 460 towards 400 mμ takes place. On the basis of Krisman's (1962) results, it can be assumed that a shortening of external branches has occurred. It is pertinent to observe that at this stage, when the iodine spectra of egg and liver glycogens become similar, we are dealing in both cases with actively metabolized glycogens. Oocyte glycogen, on the other hand, with a λ_{max} at 460 mμ, should be characterized by a relatively slow turnover rate. This last assumption finds some additional support in the following facts: (1) The apparent stability of glycogen level, even during egg segmentation (Barbieri and Gil, 1962); (2) the low respiratory activity with a R.Q. about 0.6 (Legname and Barbieri, 1962); (3) the negligible amounts of lactic acid contained in normal eggs (Barbieri and

Salomón, 1963). The apparent lack of activity exhibited by this cell and in this conection the widespread idea of viewing the unfertilized egg as an "anesthetized cell" (Brachet, 1960) should also be taken into account. Therefore, the iodine method seems to reveal, in the unfertilized egg of *B. arenarum,* the presence of a more "complete" or nearly "untouched" glycogen molecule.

We have found that glycogen isolated from eggs, as well as from several other sources, exhibits a high degree of polydispersity (Staudinger, 1948; Stetten *et al.,* 1956; Manners, 1957; Barber *et al.,* 1965; Mordoh *et al.,* 1966). The fact that most of its molecules, independently of their size, seem to be simultaneously metabolized, as shown by the iodine method, also agrees with previously reported results (Stetten and Stetten, 1960; Barber *et al.,* 1965).

Concerning our first observations in connection with the enzymes involved in the breakdown of egg glycogen we have established the presence of an enzyme with the properties of a mammalian α-amylase. Although only crude preparations have been used, some chromatographic controls of the reaction products, as well as the requirement of chloride ions, seem to exclude the presence of a relatively important glucosidase or phosphorylase activity in our working conditions.

It has already been mentioned that a β-amylasic activity has been described in eggs of *Rana esculenta* and *Bufo vulgaris* (Urbani, 1962), although no conclusive evidence has been provided. It is not unlikely that what has been taken for a β-amylase was really an α-amylase. During short incubation periods, such as those utilized by the Italian authors, α-amylase attacks only the outer branches of glycogen, with the formation of linear oligosaccharides (Olavarría and Torres, 1962). This means that during this first step of enzyme action, the analytical methods employed would not allow a clear-cut distinction between the two amylolytic pathways. Besides, while α-amylases exhibit a pH optimum between 6 and 7 and β-amylases an optimum below pH 6 (Fischer and Stein, 1960; French, 1960), "both" amylases of *B. vulgaris* eggs were found to have the same optimum at pH 7.2 (Scollo Lavizzari, 1956). We have also shown that the enzymatic activity of *B. arenarum* eggs remains unaffected in the presence of 10^{-5} *M* $HgCl_2$, which is known to inhibit β-amylase activity at that concentration. Finally, for the time being and at the present stage of our knowledge, β-amylases should be circumscribed to the plant kingdom (French, 1960).

A point of special interest concerns the intracellular localization of this enzyme. Løvtrup (1955), in *Amblystoma mexicanum* eggs, as well as Urbani and collaborators, in eggs of Anura, have found that the amylolytic activity should not be linked to yolk. Contrarily, as it has been shown, in the eggs of *B. arenarum* it should be associated to particles sedimenting at the same rate as the yolk platelets of minor size. This disagreement could be explained by the extreme lability of the enzyme-particle association. In fact, we have shown that if *B. arenarum* eggs are homogenized in water, as by the above-mentioned authors, most of the enzymatic activity remains in the supernatant after sedimentation of nuclei, yolk and pigment. In this connection, it is interesting to point out that when Urbani and Scollo Lavizzari (1955) measured amylase activity on parts of dissected embryos in the tail-bud stage they found the greatest activity in the portion richest in yolk; but as they found no activity in isolated yolk platelets, their conclusion was that the enzyme was localized in the protoplasm of the vitelline cells.

The observation that amylase seems to be progressively released from its supporting particle in the course of development has a promissory value concerning the intracellular localization of its substrate. Thus, on the assumption that glycogen is not linked to particles of fraction I, it is tempting to speculate that we are dealing with a regulatory mechanism of enzyme activity based on the spatial orientation of enzyme and substrate. It is true that glycogen could also be linked, in a very labile fashion, to the same particle to which amylase appears associated, but we do not count as yet with any experimental evidence supporting this possibility. Besides, there are reasons to believe that this regulatory mechanism is operative *in vivo:* (1) At the beginning of development, amylase activity appears restricted to fraction I; (2) as the activity of this fraction decreases throughout development, the activity of the supernatant increases (total activity remaining the same); (3) only one enzyme is involved in both fractions as far as we can judge, considering the properties analyzed in the present work; and (4) the changes suffered by glycogen, as shown by the iodine method, were the same after being attacked *in vivo* and *in vitro.*

Løvtrup (1955) has found that amylase activity in the non-yolk fraction of eggs of *Amblystoma* seems to increase through the stage of gastrulation, remaining unchanged once neurulation sets in. If we suppose that in these eggs the enzyme is linked to some particulate elements and that homogenization leads to a partial detachment of the former, we may assume that Løvtrup's graph representing "amylase synthesis" actually is an expression of the passage of the enzyme from the particles to the supernatant.

A regulatory enzymatic mechanism of this kind, depending upon the spatial orientation of enzyme and substrate, has already been proposed to explain the control of respiration in amphibian eggs (Spiegelman and Steinbach, 1945). It is not unlikely that such a mechanism was more generalized in these eggs than is currently believed. In this sense, our results give a new support to the view that yolk, more than as a simple reservoir of materials for the building up of the embryo, might function as an active part in metabolic control (Barth and Barth, 1954; Wallace, 1961).

Taking into account our limited knowledge about the function of amylase in adult tissues, as well as the important role that it seems to play in the breakdown of amphibian glycogen, further investigations along this line are being programmed.

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Summary

1. An enzyme with the properties of a mammalian α-amylase in the eggs of the toad *Bufo arenarum* is described. The enzyme appears associated in a very labile fashion to particles sedimenting at 1500 g for 20 minutes in 0.25 M sucrose solution.

2. Egg glycogen is polydisperse and does not seem to be linked to the same particles to which amylase appears associated. At the beginning of development it reacts with iodine in a different way than liver glycogen of the same species, as

was shown by their absorption spectra; after gastrulation, when glycogen is supposed to be actively metabolized, the spectra of embryo and adult glycogens become similar.

3. Some evidence is presented indicating that egg glycogen in the course of development is degraded by the action of amylase, which would be progressively released from its compartment.

LITERATURE CITED

Archibald, A. R., I. D. Fleming, A. M. Liddle, D. J. Manners, G. A. Mercer and A. Wright, 1961. α-1,4-Glucosans. Part XI. The absorption spectra of glycogen- and amylopectin-iodine complexes. *J. Chem. Soc.*, 1183–1190.

Ashwell, G., 1957. Colorimetric analysis of sugars. *In:* Methods in Enzymology, edited by S. P. Colowick and N. O. Kaplan, Academic Press, New York.

Bailey, J. M., and W. J. Whelan, 1961. Physical properties of starch. I. Relationship between iodine stain and chain length. *J. Biol. Chem.*, **236**: 969–973.

Barber, A. A., W. W. Harris and G. M. Padilla, 1965. Studies of native glycogen isolated from synchronized *Tetrahymena pyriformis* (HSM). *J. Cell Biol.*, **27**: 281–292.

Barbieri, F. D., and J. A. Gil, 1962. Utilización del glucógeno en huevos en desarrollo de *Bufo arenarum*. *Arch. Bioq. Quim. Farm.—Tucumán*, **10**: 41–47.

Barbieri, F. D., and H. Salomón, 1963. Some experimental studies on glycogen and lactic acid in the developing eggs of *Bufo arenarum*. *Acta Embryol. Morphol. Exper.*, **6**: 304–310.

Barth, L. G., 1946. Studies on the metabolism of development. *J. Exp. Zool.*, **103**: 463–486.

Barth, L. G., and L. J. Barth, 1954. The Energetics of Development. Columbia University Press, New York.

Boell, E. J., 1955. Energy exchange and enzyme development during embryogenesis. *In:* Analysis of Development, edited by B. H. Willier, P. A. Weiss and V. Hamburger, W. B. Saunders Co., Philadelphia.

Brachet, J., 1934. Étude du métabolisme de l'oeuf de grenouille (*Rana fusca*) au cours du développement. I. La respiration et la glycolyse, de la segmentation à l'éclosion. *Arch. Biol.*, **45**: 611–727.

Brachet, J., 1960. The Biochemistry of Development. Pergamon Press, London.

Brachet, J., and J. Needham, 1935. Étude du métabolisme de l'oeuf de grenouille (*Rana fusca*) au cours du développement. IV. La teneur en glycogene de l'oeuf de la ségmentation à l'éclosion. *Arch. Biol.*, **46**: 821–835.

Cohen, A. I., 1954. Studies on glycolysis during the early development of the *Rana pipiens* embryo. *Physiol. Zoöl.*, **27**: 128–141.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**: 350–356.

Fischer, E. H., and E. A. Stein, 1960. α-Amylases. *In:* The Enzymes, edited by P. D. Boyer, H. Lardy and K. Myrbäck, Academic Press, New York.

French, D., 1960. β-Amylases. *In:* The Enzymes, edited by P. D. Boyer, H. Lardy and K. Myrbäck, Academic Press, New York.

Gregg, J. R., 1948. Carbohydrate metabolism of normal and of hybrid amphibian embryos. *J. Exp. Zool.*, **109**: 119–134.

Gregg, J. R., and S. Løvtrup, 1950. Biochemical gradients in the axolotl gastrula. *C. R. Lab. Carlsberg, Ser. Chim.*, **27**: 307–324.

Gregg, J. R., J. J. MacIsaac and M. A. Parker, 1964. Anaerobic glycolysis in amphibian development. Homogenates. *Biol. Bull.*, **127**: 256–270.

Heatley, N. G., and P. E. Lindahl, 1937. Studies on the nature of the amphibian organization center. 5. The distribution and nature of glycogen in the amphibian embryo. *Proc. Roy. Soc. London, Ser. B.*, **122**: 395–402.

Jaeger, L., 1945. Glycogen utilization by the amphibian gastrula in relation to invagination and induction. *J. Cell. Comp. Physiol.*, **25**: 97–120.

KRISMAN, C. R., 1962. A method for the colorimetric estimation of glycogen with iodine. *Anal. Biochem.*, **4**: 17–23.

LEGNAME, A. H., AND F. D. BARBIERI, 1962. El metabolismo respiratorio en el desarrollo inicial de *Bufo arenarum*. *Arch. Bioq. Quim. Farm.—Tucumán*, **10**: 31–39.

LØVTRUP, S., 1955. Chemical differentiation during amphibian embryogenesis. *C. R. Lab. Carlsberg, Sér. Chim.*, **29**: 261–314.

MANNERS, D. J., 1957. The molecular structure of glycogens. *Advances in Carbohydrate Chemistry*, **12**: 261–298.

MORDOH, J., C. R. KRISMAN AND L. F. LELOIR, 1966. Further studies on high molecular weight liver glycogen. *Arch. Biochem. Biophys.*, **113**: 265–272.

OLAVARRÍA, J. M., AND H. N. TORRES, 1962. Mechanism of action of liver α-amylase. *J. Biol. Chem.*, **237**: 1746–1751.

PISANÓ, A., 1956. Método para mantener la hipófisis de anfibio fisiológicamente "in vitro." *Arch. Farm. Bioq.—Tucumán*, **7**: 387–392.

RAVEN, CH. P., 1935. Experimentelle Untersuchungen über den Glycogenstoffwechsel des Organisationszentrums in der Amphibiengastrula. II. *Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam*, **38**: 1107–1109.

SCOLLO LAVIZZARI, G., 1956. Il comportamento delle alfa e beta amilasi durante lo sviluppo embrionale e larvale di *Bufo vulgaris*. *Ric. Scient.*, **26**: 2146–2152.

SCHLAMOWITZ, M., 1951. On the nature of rabbit liver glycogen. II. Iodine absorption spectrum. *J. Biol. Chem.*, **190**: 519–527.

SPIEGELMAN, S., AND H. B. STEINBACH, 1945. Substrate-enzyme orientation during embryonic development. *Biol. Bull.*, **88**: 254–268.

STAUDINGER, H., 1948. Über natürliche Glykogene. *Makromol. Chem.*, **2**: 88–108.

STETTEN, D. W., AND M. R. STETTEN, 1960. Glycogen metabolism. *Physiol. Revs.*, **40**: 505–534.

STETTEN, M. R., H. M. KATZEN AND D. STETTEN, 1956. Metabolic inhomogeneity of glycogen as a function of molecular weight. *J. Biol. Chem.*, **222**: 587–599.

SWANSON, M. A., 1948. Studies on the structure of polysaccharides. IV. Relation of the iodine color to the structure. *J. Biol. Chem.*, **172**: 825–837.

THOMA, J. A., AND D. FRENCH, 1960. The starch-iodine-iodide interaction. Part 1. Spectrophotometric investigations. *J. Amer. Chem. Soc.*, **82**: 4144–4147.

URBANI, E., 1962. Comparative biochemical studies on amphibian and invertebrate development. *In*: Advances in Morphogenesis, edited by A. Abercrombie and J. Brachet, Academic Press, New York.

URBANI, E., AND G. SCOLLO LAVIZZARI, 1955. Studio delle amilasi nello sviluppo embrionale e larvale di *Bufo vulgaris*. *Ric. Scient.*, **25**: 1151–1159.

WALLACE, R. A., 1961. Enzymatic patterns in the developing frog embryo. *Develop. Biol.*, **3**: 486–515.

WOERDEMANN, M. W., 1933. Ueber den Glycogenstoffwechsel des Organisationszentrums in der Amphibiengastrula. *Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam*, **36**: 189–193.